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Perspective

Different strategies for producing naturally soluble form of common cytokine receptor γ chain

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ABSTRACT

The common cytokine receptor γ chain (γc) plays an essential role in regulating lymphoid homeostasis. In fact, alteration of this gene causes severe immunodeficiency in humans and animals. Although soluble γc (sγc) was identified in the late 1990s, much remains unknown about its production. This study describes various mechanisms underlying the generation of sγc isoforms in different species. Our data demonstrate that mouse γc and the avian ortholog γc-a did not generate sγc. Moreover, two mouse isoforms, CRA-a and mγc-b, encoded by transcripts lacking a transmembrane region by alternative splicing, did not yield sγc. However, in ducks, sγc was produced from a γc-b transcript lacking a transmembrane region by alternative splicing. In chickens, sγc was produced in normal cells and cell lines by proteolytic shedding of the γc-b isoform containing intron 5, which displayed a relatively high probability of proteolytic cleavage of the ectodomain. This shedding was suppressed by leupeptin, serine and cysteine protease inhibitor. Compared to the chicken ortholog γc-a, expression of γc-b mRNA was differentially regulated according to tissue type, developmental stage, and antigen stimulation. These data demonstrate several mechanisms for producing sγc and suggest a potential role for sγc in avian lymphoid homeostatic responses to environmental antigens.

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1. Introduction

The common cytokine receptor γ chain (γc), which is also known as interleukin-2 receptor (IL-2Rγ) or CD132, is a subunit shared by receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. A member of the type 1 cytokine receptor superfamily, γc is composed of four α-helical bundles, characteristic spacing of four conserved cysteine residues, and a WSXWS motif (Alves et al., 2007; Malek and Castro, 2010; Rochman et al., 2009). γc expression is observed on B cells, T cells, natural killer (NK) cells, monocytes/macrophages, granulocytes, and dendritic cells. γc forms heterodimeric or heterotrimeric complexes with specific receptor subunits, resulting in increased ligand affinity, receptor internalization, and signal transduction (Rochman et al., 2009; Sugamura et al., 1995; Walsh, 2012). The cytoplasmic domain of γc contributes to intracellular signaling by interacting with Janus kinase (JAK) and signal transducer and activator of transcription (STAT) proteins, which ultimately regulate lymphocyte development, proliferation, and homeostasis in innate and adaptive immunity (Overwijk and Schluns, 2009; Vigliano et al., 2012; Walsh, 2012). Not surprisingly, alteration of the gene encoding γc causes X-linked severe combined immunodeficiency in humans and animals (Henthorn et al., 1994; Kovanen and Leonard, 2004; Noguchi et al., 1993).

The soluble forms of several cytokine and growth factor receptors play key roles in regulating cytokine-dependent biological activities by binding target ligands present in the bodily fluids of humans and animals (Fernandez-Botran et al., 1996; Levine, 2008; Rose-John and Heinrich, 1994). Two major mechanisms have been described for producing these soluble cytokine receptors, namely sheddase-mediated proteolytic cleavage of membrane-associated receptors and synthesis by alternative splicing of mRNA transcripts lacking a transmembrane region (Fernandez-Botran et al., 1996; Levine, 2008). While the soluble IL-6 receptor (sIL-6R) can be produced via both these mechanisms in humans, sIL-6R can only

Abbreviations: γc, common cytokine receptor γ chain; sγc, soluble γc; chγc-a, chicken γc-a; chγc-b, chicken γc-b; duγc-a, duck γc-a; duγc-b, duck γc-b; mγc, mouse γc.

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be generated by proteolytic cleavage in mice (Rose-John, 2012). In addition, alternative splicing alone has been shown to generate several soluble CD40 receptors (Eshel et al., 2008).

The gene encoding γ_c is composed of eight exons and seven introns, resulting in a 64-kDa transmembrane glycoprotein (Min et al., 2002; Sugamura et al., 1995). Soluble γ_c ($s\gamma_c$), in a limited number of cases, has been detected in sera from patients with inflammatory bowel disease (Nielsen et al., 1998), in synovial fluid from rheumatoid joints (Nishio et al., 2001), in sera from certain inbred mice, and stimulated immune cells (Meissner et al., 2001). However, $s\gamma_c$ forms were not detected in supernatants from normal and activated lymphocyte cultures or in sera from healthy individuals and patients with various disorders (Lundin et al., 2002). Despite extensive knowledge about the function of γ_c at the molecular level, the mechanisms leading to the natural production of $s\gamma_c$ remain to be elucidated. Our previous studies revealed that avians express two different γ_c transcripts on normal cells due to alternative splicing, resulting in chγ_c-b containing an in-frame 78 bp insertion between Gly-222 and Val-223 of the chγ_c-a sequence and in duγ_c-b containing a frame-switching 88-bp insertion which produced a receptor molecule lacking a transmembrane region (Jeong et al., 2011; Min et al., 2002). These findings suggest that different species produce $s\gamma_c$ isoforms via different mechanisms. Here, we demonstrate proteolytic shedding and alternative splicing as mechanisms for the generation of naturally occurring $s\gamma_c$ in normal conditions.

2. Materials and methods

2.1. Animals, treatments, and infection

Eggs from ROSS chickens were obtained from Samhwa (Chungnam, Korea) and hatched at the Gyeongsang National University. Chickens were given unlimited access to feed and water. Constant light was provided for the duration of the experiments. Ten-day-old chickens were orally infected with 1×10^4 sporulated *E. tenella* oocysts (Korean isolate 291-7) (Jeong et al., 2012) and transferred to disposable cages (Yoo et al., 2011). *E. tenella* oocysts were cleaned by flotation on 5.25% sodium hypochlorite and washed three times with phosphate-buffered saline (PBS). Spleen and cecal tonsil were collected on days 0, 1, 4, 7, and 10 post-infection. For antigen inoculation, 3-week old chickens were injected with LPS (500 μg/kg) or ConA (500 μg/kg) via wing vein and spleen tissues collected 4 h later. Control chickens were inoculated with the same volume of PBS. Dexamethasone inoculation was performed as previously described (Kong et al., 2002). Briefly, 4-week-old chickens were administered dexamethasone (5 mg/kg) daily via intramuscular injection into the thigh for 1 week. Thereafter, spleen and thymus were collected. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Gyeongsang National University, Jinju, Republic of Korea (Approval Number: GNU-120615-C0022).

2.2. Cell culture

The chicken lymphoblast cell lines CU91 and CU205 (Schat et al., 1992), chicken B cell line DT40 (Baba et al., 1985), macrophage cell line HD11 (Beug et al., 1979), COS-7 cells, splenic lymphocytes, and peripheral blood mononuclear cells (PBMC) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (10,000 unit/ml) (Hyclone) at 37 °C or 41 °C in 5% CO_2. Splenic lymphocytes were resuspended to 4×10^6 cells/ml and stimulated with 25 μg/ml poly I:C, 10 μg/ml lipopolysaccharide (LPS from *E. coli*, 0111:B4), 10 μg/ml lipoteichoic acid (LTA) (all purchased from Sigma-Aldrich, Germany), or 10 μg/ml concanavalin (ConA) (Amersham Bioscience, Sweden) for 0, 4, 8 and 24 h.

2.3. Plasmid construction and cell transfection

Chicken and duck γ_c-a and γ_c-b (Jeong et al., 2011; Min et al., 2002) DNA, as well as mouse γ_c DNA (Kumaki et al., 1993) were isolated by PCR from single-stranded cDNA synthesized from splenic lymphocyte mRNA using gene-specific primers with FLAG- or Myc-tagging sequence (Supplementary Table S1). Due to no available antibody against interesting proteins, adding the FLAG- or Myc-tag allows detection of the proteins by Western blotting. PCR products were digested with *Hin*dIII and *Eco*RI, cloned into the corresponding restriction sites of pcDNA 3.1 (Invitrogen, USA), and verified by DNA sequencing. COS-7 cells were transiently transfected with 10 μg of γ_c-expressing construct or empty vector (negative control) using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol. Transfected cells were cultured for 24 to 48 h in serum-free DMEM at 37 °C in 5% CO_2, and when necessary, treated with PNGase-F (NEB, USA), chloroquine, Brefeldin A (an inhibitor of protein transport from the endoplasmic reticulum to Golgi), and protease inhibitors (Sigma Aldrich, USA) as described.

2.4. Western blotting

Cells were lysed with ice-cold buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% sodium deoxycholate, 2 mM EDTA, 0.1 SDS, and 1% Triton X-100) containing 1% Halt protease inhibitor cocktail (Thermo Fisher Scientific, USA) and centrifuged at 12,000 rpm for 30 min at 4 °C to remove debris. Culture supernatants were centrifuged to remove cell debris and concentrated up to 10-fold using centrifugal filters (Merck Millipore LTD, Germany). Cell lysates and supernatants were mixed with an equal volume of reducing sample buffer (0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.004% bromophenol blue), boiled for 5 min, resolved on 10% SDS-polyacrylamide gels, and then transferred to polyvinylidene difluoride (PVDF) membranes (Biorad, USA). Membranes were blocked with PBS containing 5% nonfat dry milk for 2 h at room temperature and incubated with the appropriate primary antibody at 4 °C overnight. γM1–11 monoclonal antibody against the extracellular domain of chγ_c (Min et al., 2002), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Bioworld, USA), as well as FLAG and Myc antibodies (Cell Signaling Technology, USA) were used. After washing three times with PBS containing 0.1% Tween 20 (PBS-T), membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Promega, USA) in PBS containing 1% nonfat dry milk for 40 min at room temperature. Membranes were washed five times with PBS-T and then five times with distilled water, visualized using an Enhanced Chemiluminescence Kit and Western Blotting Detection Reagent (GE Healthcare Life Sciences, USA), and exposed to X-ray film (AGFA, Belgium) as described previously (Jeong et al., 2012).

2.5. Quantitative real-time PCR (qRT-PCR)

Tissues and cells were homogenized in RiboEx reagent (GeneAll, Korea) and total RNA was isolated using RNeasy spin columns followed by DNase treatment (Qiagen, Germany). Total RNA was measured using a nano spectrophotometer (Optizen, Korea) and cDNA synthesis was performed using the Quantitect Reverse Transcription kit (Qiagen). Real-time PCR was performed on a CFX96 real-time PCR system (Bio-Rad) with SYBR Green (Bioneer, Korea) using the primers listed in Supplementary Table S1. A melting curve was obtained at the end of each run to verify the presence of a single amplification product and no primer dimers. The relative expression levels of individual transcripts were normalized to that of β-actin with Bio-Rad CFX software. The gene expression levels were quantified using the comparative ΔCt method with β-actin as a reference

for normalization. The fold change in expression of each gene examined from *E. tenella*-infected chickens was calculated relative to their expression levels in the same tissues of uninfected chickens as described previously (Jeong et al., 2012).

2.6. Statistical analysis

Statistical significance was calculated using the Student's t-test or one-way ANOVA followed by the Dunnett multiple comparison test using InStat® software (GraphPad, USA). Data were considered statistically significant if $P < 0.05$, and are expressed as the mean ± standard error.

3. Results

3.1. Natural production of sγc forms by proteolysis in chickens

To address the possibility of a cleavage mechanism, 22-mer peptides generated from mammalian γc and avian γc-a, along with 36-mer peptides from avian γc-b, were analyzed using a predictive program (NetChop 3.0) for proteasomal cleavage sites (Nielsen et al., 2005). The highest ranked potential cleavage sites (threshold set at 0.5) were observed at the γc C-terminus of 22-mer peptides in human, mouse, and cow (Fig. 1A). However, different patterns of peptide cleavage were observed when the algorithm was applied to 22-mer peptides from the chicken ortholog γc-a (Fig. 1B) and quail ortholog γc-a (data not shown) (Jeong et al., 2011). Compared to mammalian γc and avian γc-a, a greater probability of cleavage was observed in the chicken isoform chγc-b (Fig. 1B) and quail isoform γc-b (data not shown), both of which possess an additional eight cleavage site due to the presence of intron 5 by alternative splicing (Fig. 1A and 1B). Moreover, intron 5 in chγc-b changed the predicted probability of cleavage at certain sites. More specifically, the probability of cleavage at positions G222, V223, and A224 were 0.03, 0.40, and 0.07 in chγc-a but were 0.53, 0.87, and 0.92 in chγc-b, respectively (Fig. 1B). A similar effect was observed in quail γc-b (data not shown). These data suggest that alternatively spliced isoforms (γc-b) with 26 amino acids inserted into the juxtamembrane region augment the probability of proteolytic cleavage.

To test this hypothesis, constructs expressing C-terminal FLAG-tagged murine γc (mγc) and chγc-a, and Myc-tagged chγc-b were transiently transfected into COS-7 cells and their expression was assessed by Western blot analysis (Fig. 1C). Cell lysates transfected with mγc and chγc-a constructs produced bands at 64 and 52 kDa, respectively, whereas chγc-b displayed two major bands corresponding to 19 and 55 kDa. Densitometric analysis of the two chγc-b bands revealed that the intensity of the lower band was 1.5- to 2.5-fold greater than the upper band although some variability was observed. Collectively, these data indicate that chγc-b, due to insertion of 26 amino acids with a molecular weight 2.8 kDa, is readily cleaved in unstimulated COS-7 cells. This result, unlike mγc and chγc-a, which show little basal shedding, suggests that, if these results could be extrapolated to the natural situation, chickens could generate naturally occurring sγc in the form of chγc-b.

We next investigated whether the naturally occurring sγc originated from chγc-b. Supernatant from COS-7 cells transfected with chγc-a and chγc-b was analyzed by Western blot using a monoclonal antibody (γM1–11) directed against the extracellular domain of chγc (Min et al., 2002). An approximately 40 kDa protein was detected in the supernatant of COS-7 cells transfected with chγc-b, but not chγc-a or empty vector alone (Fig. 1D). This molecular weight protein showed a similar size of full-length chγc-b, which has 42.5 kDa as predicted molecular weight (Min et al., 2002). When the supernatant of COS-7 cells transfected with chγc-b was treated with peptide-N-glycosidase F (PNGase-F), approximately 26 kDa proteins were observed, showing a much smaller size protein than that of full-length chγc-b. These results indicate that the observed secreted chγc-b represents 26 kDa of chγc-b with 14 kDa N-linked glycosylation (Fig. 1E). In addition, cell lysates and culture supernatants of COS-7 cells transfected with chγc-b resulted in minor bands at 22 (Fig. 1C) and 29 kDa (Fig. 1E). These experiments indicate that the naturally occurring sγc form is mainly generated from isoform chγc-b under normal conditions via cleavage at a minimum of two sites within its juxtamembrane region.

Generally, ectodomain shedding of transmembrane receptors at the cell surface occurs by proteolysis. However, studies have also demonstrated intracellular proteolytic cleavage (Keller et al., 2006; Levine, 2008). Previous reports have shown that protease and kinase inhibitors do not suppress ectodomain shedding of mγc (Meissner et al., 2001). Following endocytosis, most γc is sorted to lysosomes where it is degraded (Hémar et al., 1995). Therefore, we investigated whether the intracellular fragment corresponding to 19 kDa (Fig. 1C) is generated by spontaneous lysis within the lysosomal compartment. COS-7 cells transiently transfected with chγc-b-expressing constructs were cultured for 24 h and then treated with 200 μm chloroquine, an inhibitor of lysosomal activity. As shown in Fig. 2A, chloroquine treatment did not prevent the generation of intracellular chγc-b fragments. Treatment with brefeldin A also had no effect. These results suggest that sγc shedding from chγc-b is not linked to lysosomal proteolysis or the secretory pathway from the Golgi complex to the cell membrane. Therefore, ectodomain shedding of chγc-b most likely occurs at the cell surface.

To confirm this, COS-7 cells were transfected with a construct expressing Myc-tagged chγc-b and then treated with a cocktail of protease inhibitors, including aprotinin, bestatin, E-64, leupeptin, and pepstatin A (Fig. 2B). Of these protease inhibitors, leupeptin specifically inhibited the chγc-b cleavage in dose-dependent manners (Fig. 2C and 2D). These observations indicate that sγc generated from chγc-b is released via proteolytic cleavage at the cell surface.

3.2. Detection of naturally produced sγc in normal cells and cell lines

Quantitative real-time PCR (qRT-PCR) analysis was used to examine the expression of chγc-a and chγc-b transcripts in four chicken cell lines. The chγc-a and chγc-b transcripts were abundantly detected in CU91, CU205, and HD11 cells, whereas DT40 cells expressed much lower levels of chγc-a and chγc-b mRNA. These transcripts were not detected in COS-7 cells, which were used as a negative control (Fig. 3A). As expected, Western blotting detected specific bands corresponding to chγc-a and chγc-b protein in lysates and cell culture supernatants from CU91, CU205 and HD11, but not in DT40 and COS-7 (Fig. 3B and 3C). Additionally, culture supernatant from splenic mononuclear cells (SMC) of normal chickens contained these proteins. Similar to Fig. 2A, a 26 kDa protein was detected in the culture supernatants of HD11 and CU91 cells treated with PNGase-F (data not shown). These results indicate that chicken cells express naturally produced sγc forms under normal conditions.

3.3. Comparative analysis of chγc-a and chγc-b expression under normal, activated, and immunosuppressed conditions

The expression level of chγc-b to chγc-a (chγc-b/chγc-a) was between 8% and 21% in normal tissues from ten-day-old chickens. The expression of chγc-b was relatively high in thymus and lung, but low in heart and liver (Fig. 4A). In a day 17 embryo (E17), the level of chγc-b/chγc-a in thymus, bursa, and spleen was 25%, 17%, and 46%, respectively. However, dramatically reduced chγc-b expression was observed in the spleen after E17 (Fig. 4B).

Next, we assessed whether mitogen treatments, parasite infection, and immunosuppressant agents affect the level of

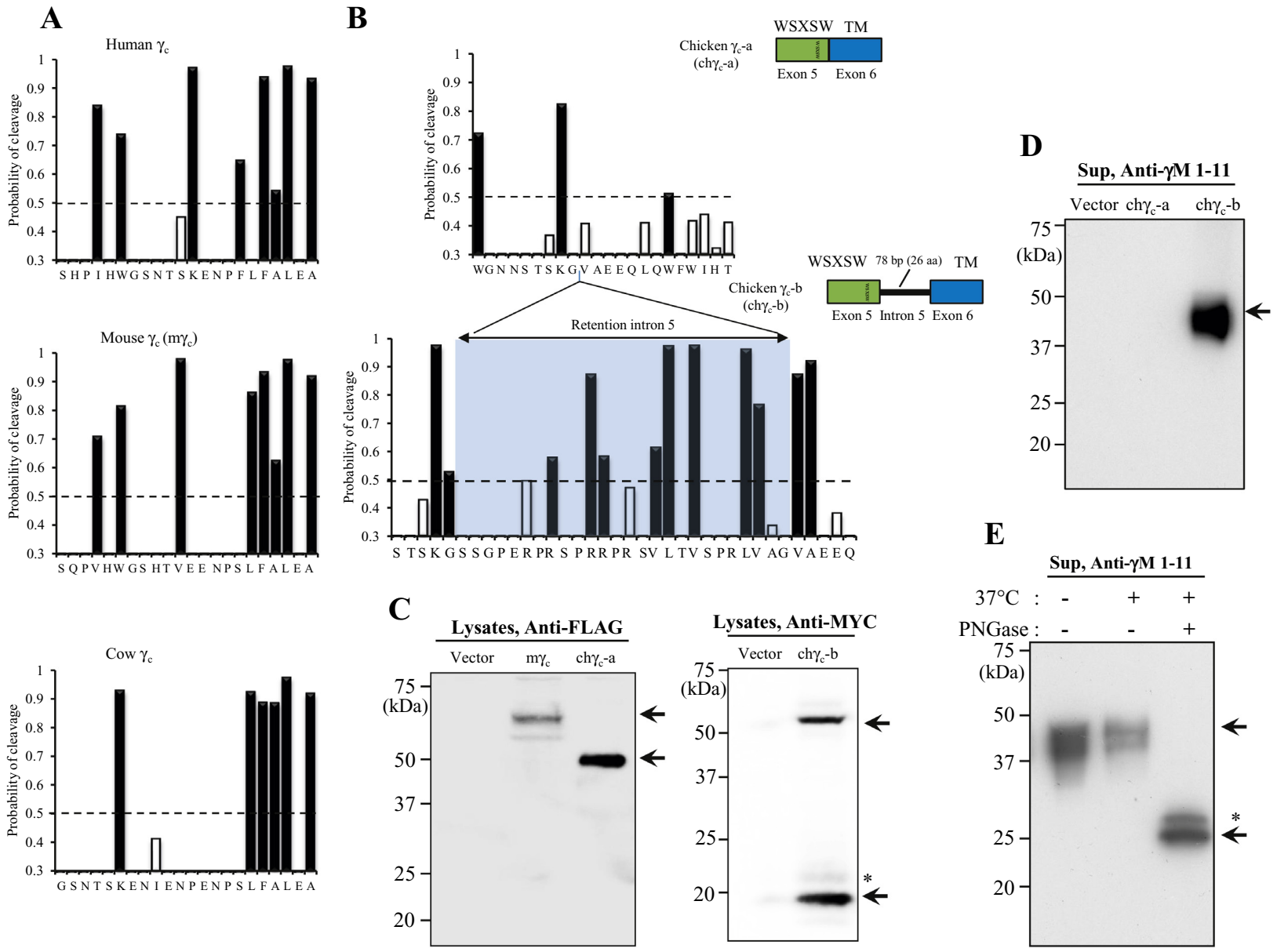

Fig. 1. Cleavage predictions of chγc-b and sγc production from chγc-b in COS-7 cells. **A, B.** The probability of cleavage surrounding the juxtamembrane region of γc in mammals (A), as well as γc-a (chγc-a) and γc-b (chγc-b) in chickens (B), in the presence of intron 5 were analyzed using the NetChop 3.0 C-terminus peptide processing algorithm. The predictive program recommended a probability of cleavage above a 0.5 threshold (black bars). Low probabilities of cleavage (below 0.5) are presented in white bars. TM, transmembrane region; WSXSW, WSXSW motif. **C, D.** Lysates (C) and supernatants (D) of COS-7 cells transiently transfected with C-terminal FLAG-tagged mouse γc (mγc) and chγc-a, and Myc-tagged chγc-b constructs were separated for Western blots by SDS-PAGE under reducing conditions. The specific bands were detected with the anti-tag or γM1–11 antibodies. γM1–11 is a monoclonal antibody directed against the extracellular domain of chγc (Min et al., 2002). Arrows indicate specific bands representing mγc or chγc. Asterisks indicate minor bands of chγc-b. Data are representative of three independent experiments with similar results. **E.** The culture supernatants (50 μl) were deglycosylated by 100 U peptide-N-glycosidase F (PNGase-F) at 37 °C for 2 h and specific bands were detected with the γM1–11 monoclonal antibody. Arrows and asterisk indicate soluble chγc and minor bands of soluble chγc, respectively.

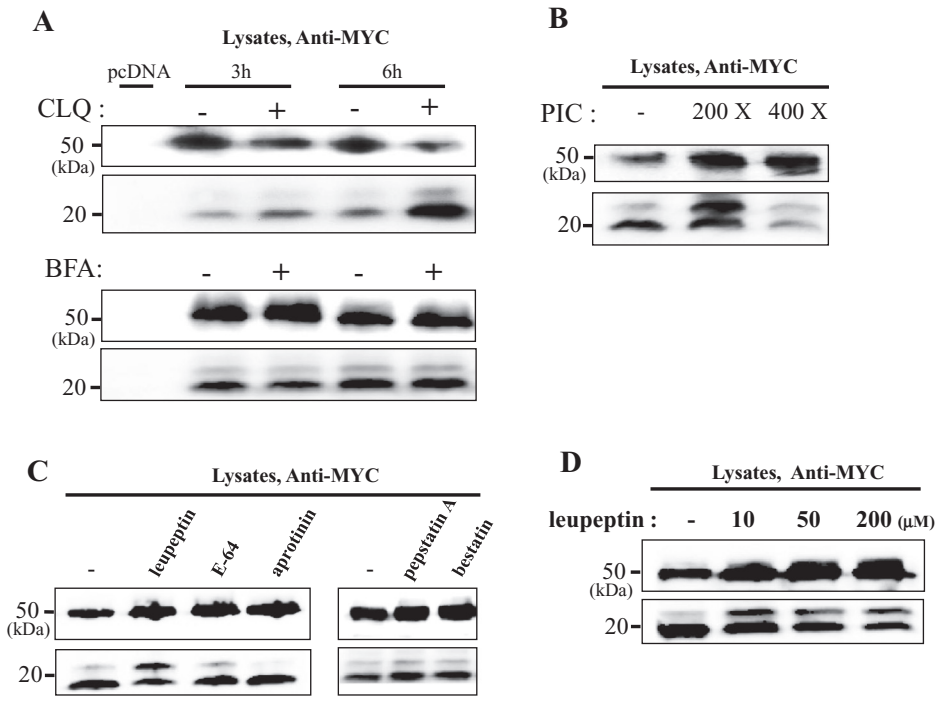

Fig. 2. Proteolytic ectodomain shedding of chγc-b at the cell surface. COS-7 cells were transiently transfected with a construct expressing Myc-tagged chγc-b for 24 h. **A.** Transfected cells were treated with 200 μM chloroquine (CLQ) or 5 μg/ml brefeldin A (BFA) for 3 and 6 h, followed by Western blot analysis using the anti-Myc antibody. **B.** Transfected cells were treated with the indicated concentrations of protease inhibitor cocktail (PIC) for 24 h, followed by Western blot analysis using the anti-Myc antibody. **C.** Transfected cells were treated with 50 μM leupeptin, 5 μM E-64, 100 μg/ml aprotinin, 5 μM pepstatin A or 100 nM bestatin for 24 h as indicated, followed by Western blot analysis using the anti-Myc antibody. **D.** Transfected cells were treated with the indicated dilutions of leupeptin for 24 h, followed by Western blot analysis using the anti-Myc antibody. Data are representative of two independent experiments with similar results.

chγc-b/chγc-a *in vivo* and *in vitro* (Fig. 4C–F). Splenic lymphocytes activated with Toll-like receptor (TLR) agonists (i.e., poly I:C, LTA, and LPS) exhibited higher chγc-b/chγc-a levels compared to unstimulated splenic lymphocytes. Interestingly, ConA-stimulated splenic lymphocytes displayed an inverse expression pattern (Fig. 4C). However, the expression level of chγc-b/chγc-a *in vivo* was increased significantly in the spleen of both ConA- and LPS-inoculated groups compared to the control group (Fig. 4D). Unlike the spleen, the cecal tonsils of *E. tenella*-infected chickens showed increased chγc-b mRNA expression compared to normal animals (Fig. 4E). IL-1β and IFN-γ expressions were examined as positive controls for ConA- and LPS stimulation, and *E. tenella* infection, respectively. Animals treated with dexamethasone, an anti-inflammatory and immunosuppressive agent, exhibited no difference in chγc-b to chγc-a expression in either the spleen or thymus (Fig. 4F). However, the spleen and thymus of dexamethasone-treated animals weighed less

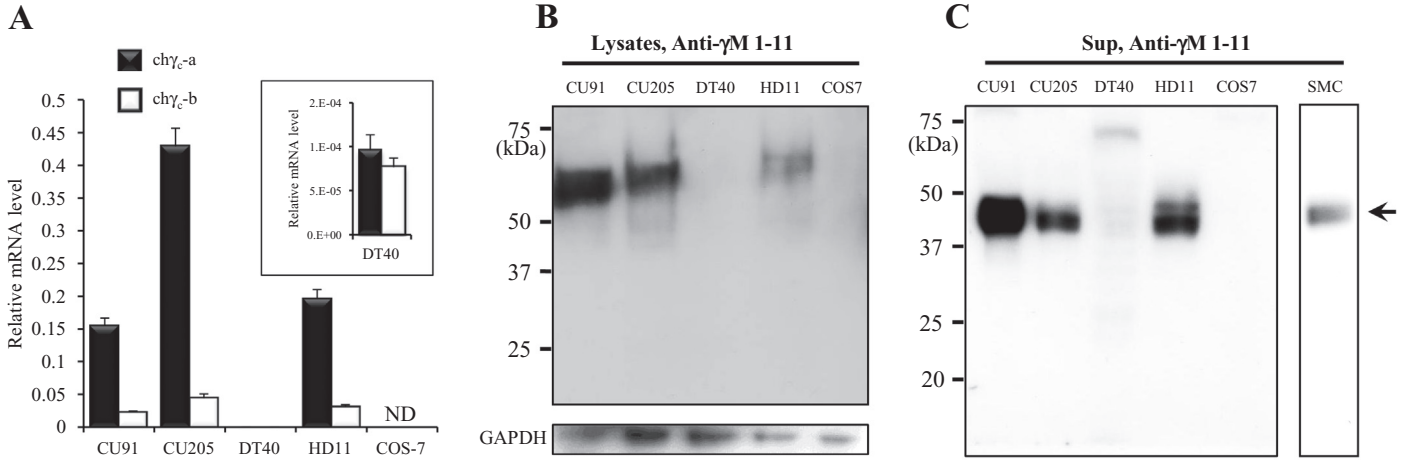

Fig. 3. Detection of naturally producing sγc in cell lines and normal splenic mononucleic cells. Expression of chγc-a and chγc-b transcripts was analyzed in various chicken cell lines and COS-7 cells by quantitative real-time PCR. **A.** Expression levels were normalized to those of β-actin from the same samples. Data represent the mean plus standard error of triplicate samples. **B, C.** Expression of chγc in whole cell lysates (B) and culture supernatants (C) from chicken lymphoid cell lines, COS-7 cells, and normal chicken splenic mononucleic cells (SMC) was assessed by Western blot using the γM1–11 monoclonal antibody. CU91 and CU205, REV-transformed lymphoblast cell lines; DT40, ALV-transformed bursal lymphoma cell line; HD11, macrophage cell line; ND, not detected. Arrows indicate soluble chγc. Data are representative of three independent experiments with similar results.

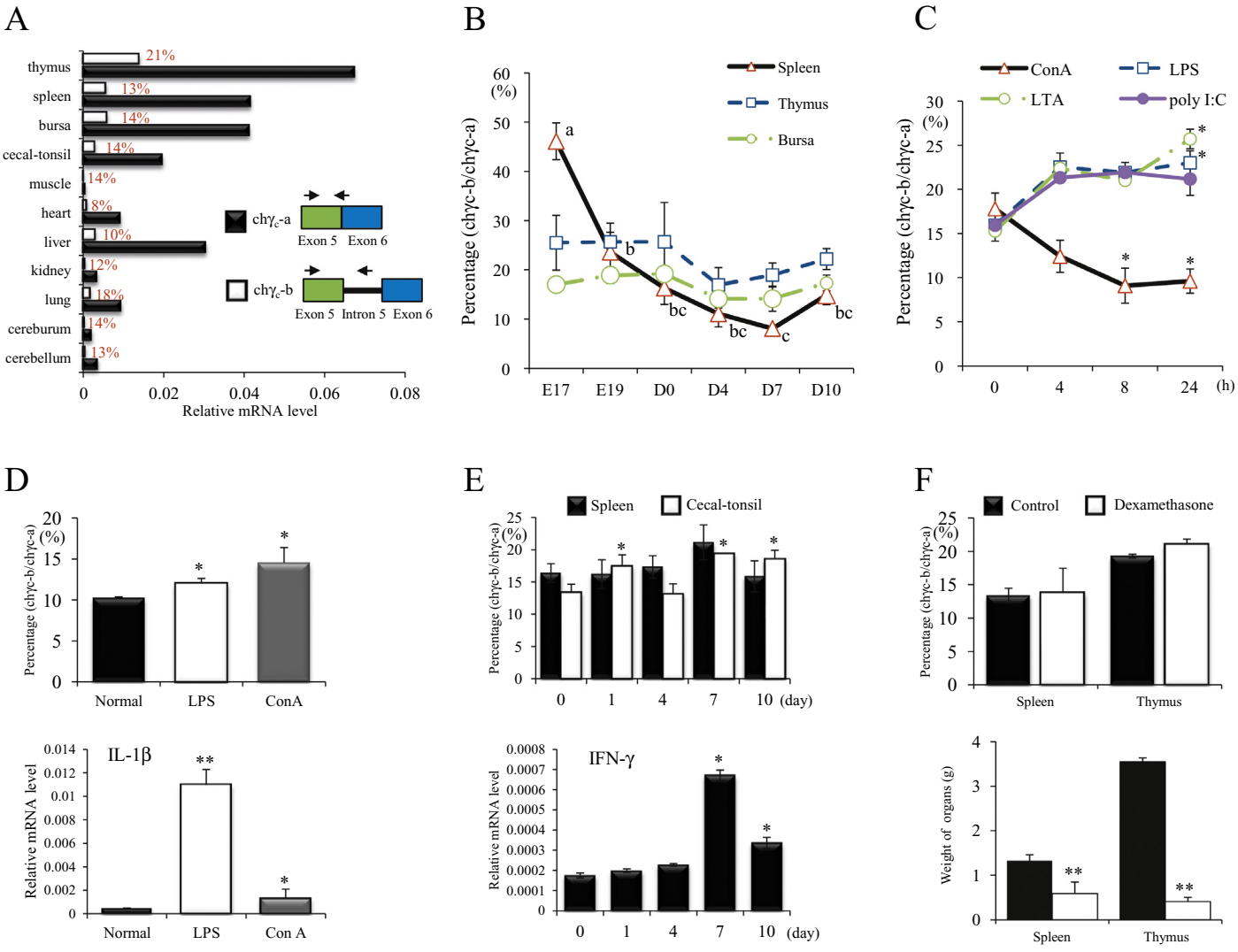

Fig. 4. Analysis of chγc-a and chγc-b expression under normal, activated, and immunosuppressed conditions. **A.** Expression levels of chγc-a and chγc-b transcripts in various chicken tissues as determined by quantitative real-time PCR (qRT-PCR). Tissue samples from 10-day-old chickens were pooled from five chickens. Percentage (%) indicates expression level of γc-b to γc-a. Location of the primers used is indicated by arrows. Each sample was analyzed in triplicate. Data are representative of two independent experiments with similar results. **B.** Expression levels of chγc-a and chγc-b transcripts at the indicated developmental stages. Tissue samples were collected at embryo days 17 (E17) and 19 (E19), and at days 0 (D0), 4 (D4), 7 (D7) and 10 (D10) post-hatch. The sample was pooled from at least five eggs or chickens. Bars represent the mean ± standard error from three independent experiments. Lowercase letters highlight statistically significant differences ($P < 0.05$) according to the Dunnett multiple comparison test. **C–F.** Effect of *in vitro* and *in vivo* stimulation on chγc-a and chγc-b expression. Splenic lymphocytes (C) were isolated from 2-week-old chickens, and then activated with 10 μg/ml ConA, 10 μg/ml LPS, 10 μg/ml LTA, or 25 μg/ml Poly I:C for the indicated times. Data represent the mean ± standard error from three independent experiments. **D.** Three-week-old chickens were injected with LPS (500 μg/kg) or ConA (500 μg/kg) into the wing vein. Control chickens were injected with an equal volume of PBS. After 4 h, the spleen was harvested and qRT-PCR was performed. Bars represent the mean ± standard error from five individual chickens. **E.** Ten-day-old chickens were orally infected with 1×10^4 sporulated *E. tenella* oocysts. Tissue samples from five chickens were collected on days 0, 1, 4, 7, and 10 post-infection and then pooled prior to performing qRT-PCR. Data represent the mean of triplicate samples, and are representative of two independent experiments with similar results. **F.** Four-week-old chickens were injected intramuscularly with dexamethasone (5 mg/kg) for 1 week and spleen and thymus were collected. Bars represent the mean ± standard error from five individual chickens. Expression levels were normalized to those of β-actin from the same samples. $^{*}P < 0.05$ or $^{**}P < 0.01$ were considered statistically significant compared to untreated controls.

compared to normal, untreated animals. These results indicate that stimulators and pathogens, but not immunosuppressant agents, increase chγc-b to chγc-a expression. Therefore, the ratio of chγc-b to chγc-a can be differentially regulated depending on the tissue type, developmental stage, and antigen stimulation.

3.4. *Ducks produce sγc by alternative splicing*

Unlike chickens, ducks most likely generate sγc by alternative splicing of mRNA transcripts lacking a transmembrane region (Jeong et al., 2011). To confirm this possibility, we examined cell lysates and culture supernatants from COS-7 cells expressing FLAG-tagged duγc-a or duγc-b for the presence of sγc isoforms. Both isoforms were present in the cell lysate (Fig. 5A). However, FLAG-tagged duγc-a, which is the duck ortholog to mammalian γc, was absent from the culture supernatant as only duγc-b was detected (Fig. 5B). The duγc-b isoform of approximately 56 kDa was sensitive to PNGase-F since its molecular weight was reduced to 42–44 kDa after treatment (Note the predicted size of duγc-b is 41.3 kDa). Moreover, addition of brefeldin A caused a dramatic increase in duγc-b expression in cell lysates, whereas sγc expression was decreased in culture supernatants (Fig. 5C). These data suggest that ducks utilize a different mechanism from chickens to generate natural sγc forms.

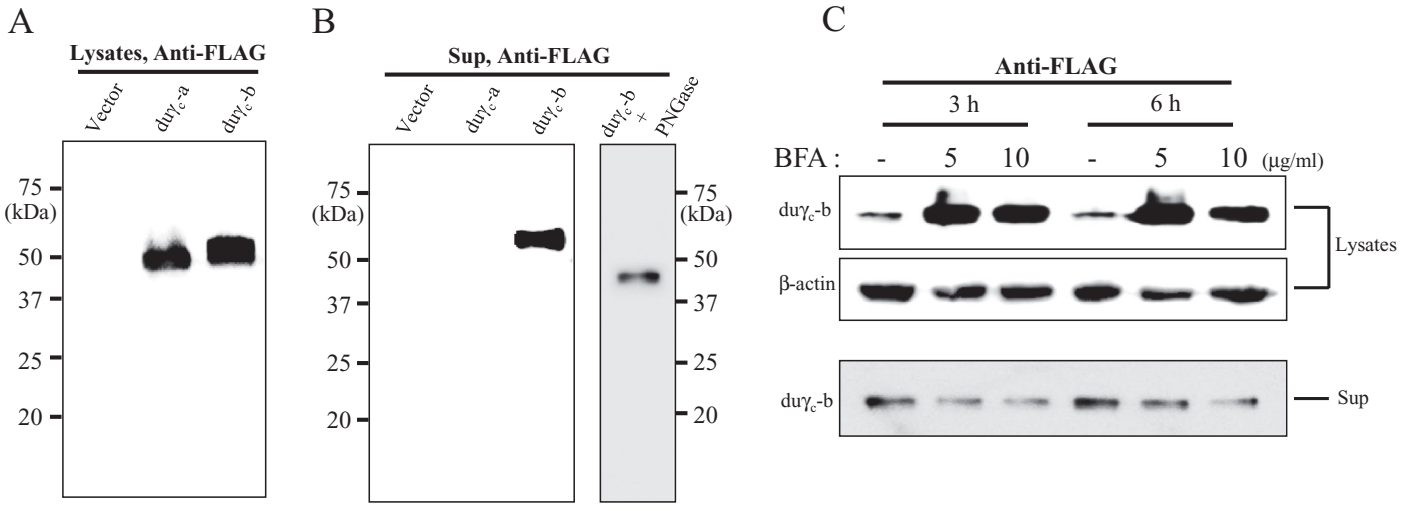

Fig. 5. Ducks produce sγc by alternative splicing. **A–C.** COS-7 cells transiently were transfected with a FLAG-tagged duγc-a or duγc-b construct for 24 h and then examined by Western blot. Lysates (A), culture supernatants (B), and cells (C) were treated with peptide-N-glycosidase F (PNGase-F) and brefeldin A (BFA) as indicated. The samples were separated by SDS-PAGE under reducing conditions and specific bands were detected with an anti-FLAG antibody. Data are representative of two independent experiments with similar results.

3.5. Mouse γc and its isoforms exist mainly in cell lysates

The presence of sγc was previously reported in sera from certain inbred mouse strains and stimulated immune cells. Interestingly, protease and kinase inhibitors did not suppress sγc production under these conditions (Meissner et al., 2001). A spliced IL-2Rγc isoform (CRA-a or sγc) lacking exon 6 harboring a transmembrane region was detected in the mouse, rat or human (Hong et al., 2014; Olosz and Malek, 2002). However, our data show that the mγc and CRA-a isoforms predominated in the lysates, but not in culture supernatants (data not shown), of COS-7 (Fig. 6D) and HeLa cells (data not shown) transfected with FLAG-tagged mγc and CRA-a constructs. Likewise, normal COS-7 cells transfected with murine γc did not produce sγc (Meissner et al., 2001). Thus, we investigated whether additional γc isoforms exist in the mouse. Interestingly, a novel γc cDNA (hereby called mγc-b) lacking part of exon 5 and the WSXWS motif, due to alternative splicing, was detected by RT-PCR in the spleen of C57BL/6, ICR (Fig. 6A and 6B), as well as Balb/c mice (data not shown). This mγc-b gene encodes a 266 amino acid protein that lacks a transmembrane region due to a frame-shifting caused by a 104-bp deletion in exon 5 (Fig. 6B and C). mγc-b isoform was mainly detected in the lysates, but not in culture supernatants (data not shown), of COS-7 (Fig. 6D) and HeLa cells (data not shown) transfected with a FLAG-tagged mγc-b construct. In addition, brefeldin A treatment did not affect the level of CRA-a or mγc-b expression in lysates (Fig. 6E), implying that mγc and two isoforms do not produce abundantly the soluble receptors.

4. Discussion

Soluble cytokine receptors regulate biological events by binding and modulating the activity of target cytokines in either an antagonistic or agonistic fashion. The two major mechanisms for producing soluble receptors, alternative splicing and proteolytic cleavage, are not mutually exclusive or species-dependent (Hayashida et al., 2010; Levine, 2008). The mechanisms underlying the generation of sγc remain to be elucidated; however, several studies have investigated the production and function of sγc (Meissner et al., 2001; Nielsen et al., 1998; Nishio et al., 2001). Here, we report novel shedding mechanisms that lead to the production of sγc.

Our previous work demonstrated that avians generate the γc-b isoform harboring the fifth intron by alternative splicing (Jeong et al., 2011; Min et al., 2002). From γc-b isoforms, chickens and ducks produce sγc by proteolytic shedding and alternative splicing that results in transcripts lacking a transmembrane region, respectively. However, mγc, as well as the chicken and duck ortholog γc-a, produced little to no sγc. Given that protease and kinase inhibitors did not affect sγc generation (Meissner et al., 2001), these data suggest that normal γc do not typically give rise to sγc except in a limited number of clinical disorders (Nielsen et al., 1998; Nishio et al., 2001) and sera from certain inbred mice (Meissner et al., 2001). Previous reports suggested that the two mouse γc isoforms, CRA-a (Olosz and Malek, 2002) and mγc-b, may produce sγc; however, our data demonstrate that they do not generate sγc in transfected COS-7 cells. Nevertheless, sγc produced from CRA-a, but not mγc-b, could be detected at very low levels with increased exposure time by Western blot (data not shown). It is noteworthy that calpains, which are calcium-dependent, non-lysosomal cysteine proteases (Saatman et al., 2010), can interact with γc to cleave intracellular PEST (proline, glutamate, serine, and threonine) motifs, suggesting that γc proteolysis by calpains could represent a regulatory mechanism for γc-dependent cytokines (Noguchi et al., 1997). Moreover, sγc was not detected in culture supernatants from normal and activated lymphocytes or in serum samples from healthy patients (Lundin et al., 2002). Taken together, these observations indicate that, unlike mice and humans, avians express abundant levels of sγc under normal conditions.

Molecules enriched from patient sera using IL-2Rα or IL-2Rβ affinity chromatography revealed the presence of proteins that bind to antibodies specific for γc (Dummer et al., 1996). Recombinant mouse sγc comprised of an extracellular WSXWS motif can inhibit cell proliferation induced by γc-dependent cytokines (Meissner et al., 2001). Thus, it can be hypothesized that chicken and duck sγc harboring an extracellular WSXWS motif possess critical biological functions. Furthermore, the cytoplasmic domain of γc contains two Src homology 2 (SH2) domains (Nelson and Willerford, 1998; Sugamura et al., 1995) that contribute to intracellular signaling by interacting with phosphotyrosine residues of various effector molecules (Malek and Castro, 2010). The intracellular region of chγc-b contains a region with limited homology to SH2 domains (Min et al.,

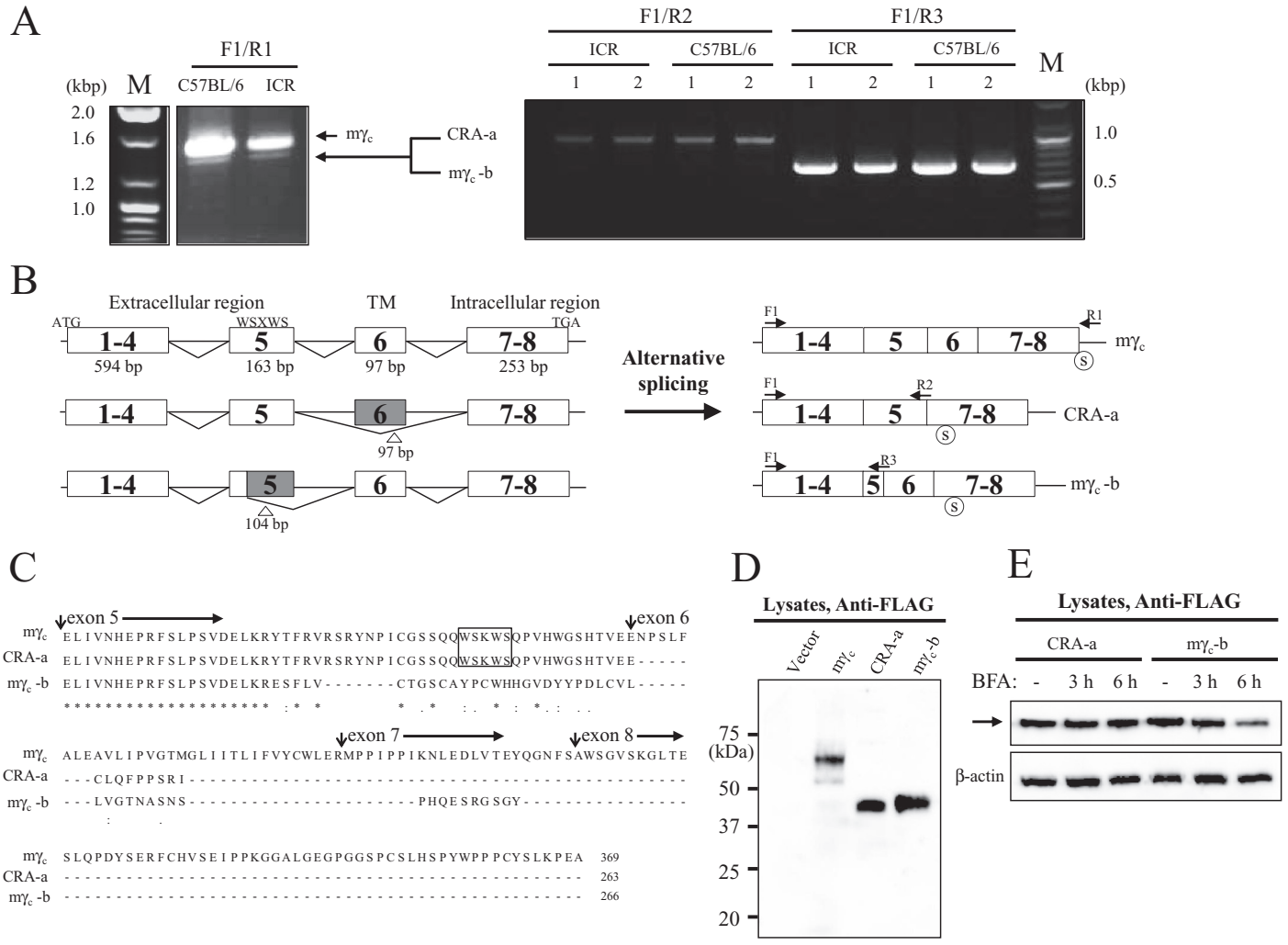

Fig. 6. Identification of mouse γc isoforms in cell lysates. **A.** A new isoform, mγc-b, was detected in different mouse species by RT-PCR. M, DNA marker. **B.** Diagram of the frame shift produced by alternative splicing that results in mγc-b generation. Arrows indicate the primers used (F1, R1, R2, R3). Position of the first ATG and the stop codon (Ⓢ) are indicated. The deleted exons are indicated by gray boxes. TM; transmembrane region, WSXSW; WSXSW motif. **C.** Multiple sequence alignment of mouse γc (mγc) and differential splicing from CRA-a and mγc-b. Sequences were aligned using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/). Accession numbers used in this comparison were mγc (NM_013563.3), CRA-a (EDL14151.1), and mγc-b (KC815469). The WSXWS motif is boxed and exon junctions are indicated by arrows. **D, E.** COS-7 cells were transiently transfected with a construct expressing FLAG-tagged mγc, CRA-a, or mγc-b for 24 h, followed by Western blot analysis with an Anti-FLAG antibody. Cells were treated with 5 μg/ml brefeldin A (BFA) for 3 or 6 h (E). Data are representative of three independent experiments with similar results.

2002). IL-2Rβ is subject to ectodomain shedding, which generates an intracellular fragment with a role in phosphorylation, association with STAT5A, and cell proliferation (Montes de Oca et al., 2010). Considering this, we propose that an intracellular 20 kDa fragment of chγc-b may be biologically functional; however, this hypothesis remains to be investigated.

Using the well-established chicken model of infection with the protozoan parasite *Eimeria* (Shirley and Lillehoj, 2012), we examined chγc-b mRNA expression in the spleen and cecal tonsils of *E. tenella*-infected chickens. Cecal tonsils, but not spleen, showed upregulation of chγc-b expression. It is interesting to note that *E. tenella* preferentially infects the cecum in a region-specific manner and induces mucosal immunity mediated by intestinal intraepithelial lymphocytes, resulting in protective immunity against infection (Min et al., 2004; Sharman et al., 2010; Shirley and Lillehoj, 2012).

Alternative splicing can alter the function of a gene in different tissues and developmental states by generating distinct mRNA isoforms (Hughes et al., 2012; Modrek et al., 2001). Comparative expression analysis of chγc-a and chγc-b mRNA revealed that chγc-b expression was 21–29% higher in the spleen versus the thymus and bursa in E17 embryos. In addition, treatment of chicken splenocytes with LPS and ConA, but not dexamethasone, led to increased chγc-b mRNA expression. All TLR agonists tested upregulated chγc-b mRNA in splenic lymphocytes, whereas ConA decreased expression. Considering that γc has multiple roles, including lymphocyte development, homeostasis, and cell proliferation (Alves et al., 2007; Overwijk and Schluns, 2009; Rochman et al., 2009; Vigliano et al., 2012), these observations suggest a role for chγc-b in lymphocyte development and/or immune responses to environmental antigens.

Although further work is needed to clarify the physiological function of sγc in regulating signaling by γc-dependent cytokines in different species, our data demonstrate that sγc are naturally produced via different mechanisms, suggesting that the sγc generated by proteolysis or alternative splicing could represent a different strategy by which cytokine function can be controlled.

Conflict of interest

The authors have no financial conflicts of interest.

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Appendix: Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2014.08.008.

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